Arimoclomol Upregulates Expression of Genes Belonging to the Coordinated Lysosomal Expression and Regulation (CLEAR) Network

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Hadeel Shammas[1], Nikolaj Havnsøe Torp Petersen[2], Pontus Klein[2], Anja Koustrup[2], Marianne Terndrup Pedersen[2], Anne Sigaard Bie[2], Travis Mickle[1], Cathrine Kolster Fog[2], Thomas Kirkegaard Jensen[2], Sven Guenther[1]

[1]Zevra Therapeutics, Celebration, FL, USA, [2]Orphazyme A/S, Copenhagen, Denmark.

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Background and Objective

Niemann-Pick disease type C (NPC) is an ultra-rare and fatal neurodegenerative disease (1:100,000 live births). It is a highly heterogeneous disease with relentlessly progressive symptoms. In NPC, normal lysosomal function is disrupted by mutations in the *NPC1* (95% of cases) or *NPC2* genes. The dysfunction of either of these genes results in a reduced amount of properly folded and mature NPC protein, or even a complete lack of NPC protein. The consequence is lysosomal dysfunction with accumulation of unesterified cholesterol in lysosomes and late endosomes, which is cytotoxic and causes neurodegeneration and peripheral organ dysfunction.

Arimoclomol, an orally available small molecule, is an FDA-approved treatment for NPC when used in combination with miglustat. The purpose of the in vitro studies was to explore the pathways by which arimoclomol targets the fundamentals of NPC etiology.

Results

The proposed mechanism of action of arimoclomol is illustrated in **Figure 1** and summarized below:

- After 1 day of treatment with arimoclomol, the translocation of TFE3 from the cytosol to the nucleus was significantly increased compared to vehicle in both healthy human and NPC patient fibroblasts across 3 genotypes containing either the I1061T or P1007A mutation (**Figure 2**).
- A dose-dependent (0–400 µM) arimoclomol-enhanced translocation of TFE3 and TFEB from the cytosol to the nucleus was demonstrated in HeLa cells treated with the NPC1 protein inhibitor U-18666A that induces an NPC like phenotype (**Figure 3**). The impaired function of NPC1 protein alone promoted some translocation of TFE3/TFEB to the nucleus indicating that the NPC phenotype is more sensitive to TFE3/TFEB activation by arimoclomol.
- Treatment with arimoclomol (400 µM) was shown to significantly enhance the binding of TFE3 to the CLEAR promoter elements of *NPC1*, *NPC2*, *GBA*, *MCOLN*, and *GLA* in healthy human fibroblasts, whereas binding of TFE3 to the negative control *ACTB* promoter was not affected by arimoclomol (**Figure 4**).
- Transcriptional upregulation with arimoclomol (400 µM) was observed for all tested CLEAR genes related to lysosomal function (*NPC1*, *NPC2*, *GBA*, *GLA*, *MCOLN1*, *RRAGD*, *SQSTM1*) in healthy human fibroblasts (data not shown).
- Arimoclomol (0–400 µM) increased NPC1 protein concentrations in all genotypes in a dose-dependent manner with the greatest effects observed in the I1061T/I1061T genotype (**Figure 5**).
- Increased maturation of mutant NPC1 proteins in the NPC fibroblasts was demonstrated in cells incubated with arimoclomol compared to untreated control by use of the endoglycosidase H (Endo H) assay (data not shown).
- Arimoclomol (100 and 200 µM) increased the cholesterol clearance from the lysosomal compartment demonstrated by reduced filipin staining intensity at prespecified timepoints (7, 14, 21, 28 days), with the most pronounced effect obtained with the highest arimoclomol dose (**Figure 6**).

Figure 1: Arimoclomol Targets the Pathophysiology of NPC by Two Pathways

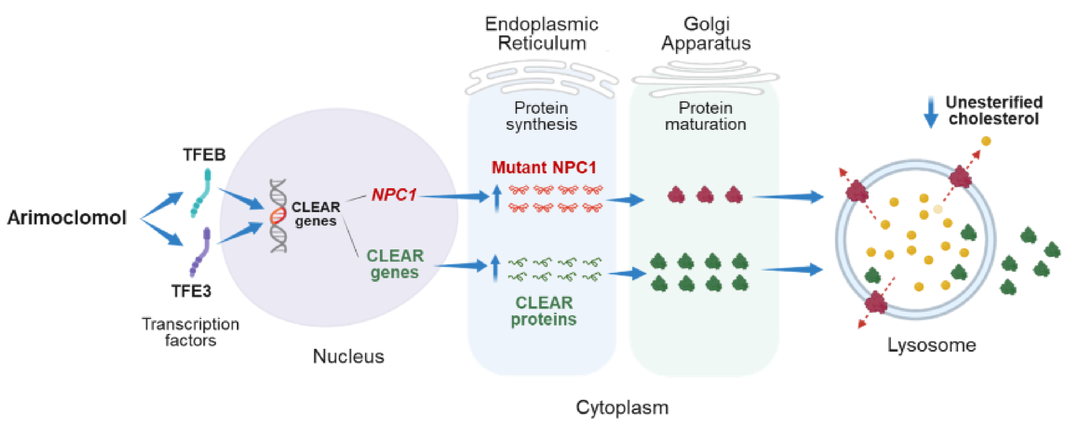
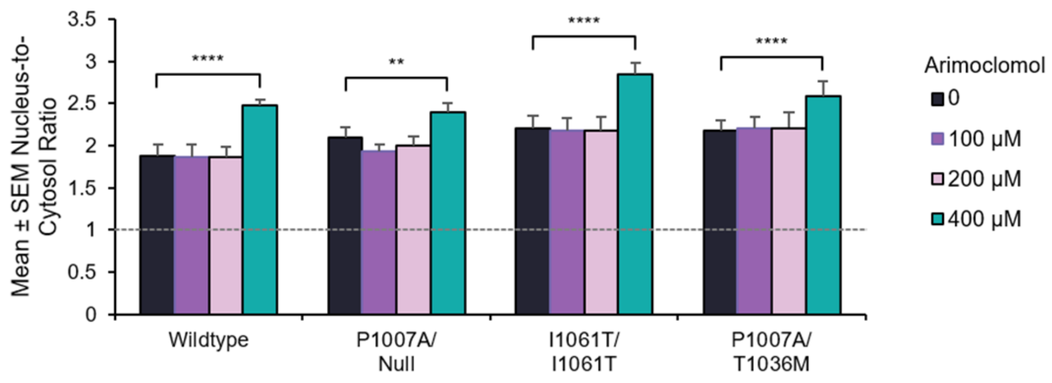
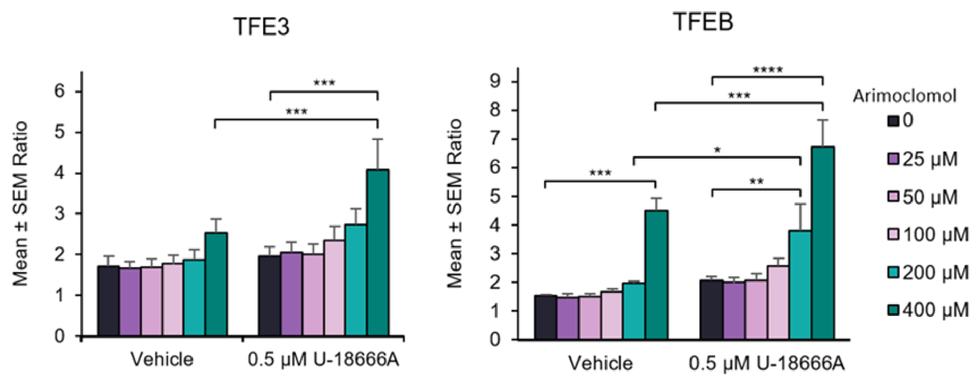


Figure 2: Arimoclomol Increases Nuclear-to-Cytoplasmic Ratio of TFE3 in Human Wild-type and NPC Fibroblasts



Immunofluorescence staining of TFE3 in NPC and wild-type fibroblasts treated with PBS (control) or 100-400 µM arimoclomol for 1 day. The experiment was repeated 3 times, each column representing more than 2300 cells in total. The mean intensity ratio of the TFE3 staining in the nuclear to cytosolic compartments per cell was quantified and displayed as a bar graph with mean + SEM.
**p <0.01, ****p<0.0001.

Figure 3: Arimoclomol Enhances Translocation of TFE3 and TFEB from Cytosol to Nucleus in HeLa cells where NPC1 Protein was Inhibited by U-18666A

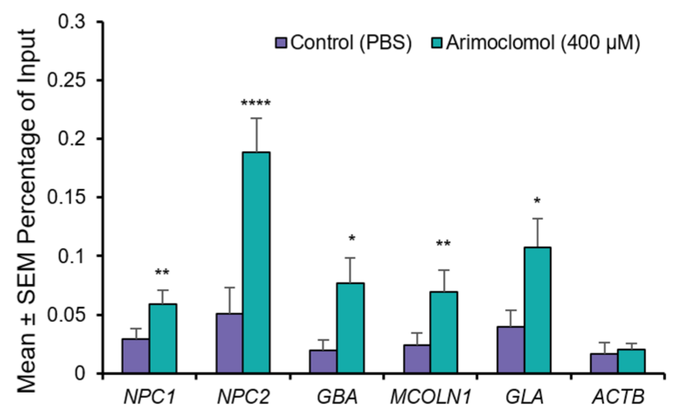


Quantifications of the nucleus-to-cytosol mean fluorescence intensity ratios of immunostainings of TFE3 and TFEB in HeLa cells. Cells were treated with arimoclomol in combination with either vehicle or 0.5 µM NPC1 inhibitor U-18666A for 3 days.
Bars depict the means of median ratios of independent experiments (n=4), each with >160 cells per sample, error bars show SEM.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Conclusions

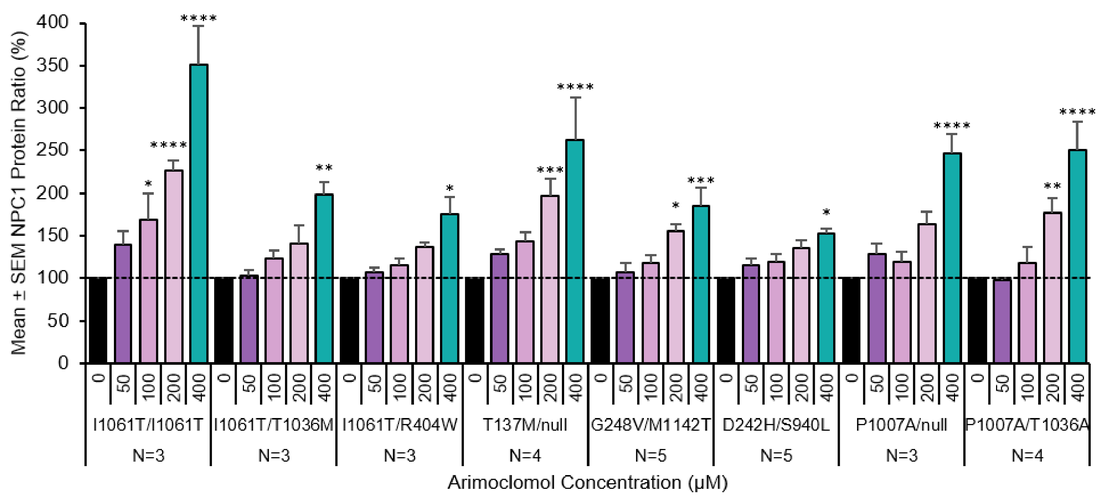
- The presented in vitro data provide mechanistic evidence of how arimoclomol can target NPC through multiple mechanistic pathways when the disease is caused by functional null mutations or missense mutations.
- Increased translocation of the transcription factors TFE3 and TFEB from the cytosol to the nucleus is a crucial step that results in upregulation of a series of downstream processes that may improve lysosomal function and cell viability.
- The data support that arimoclomol not only upregulates expression of certain CLEAR genes and specifically *NPC1* at the transcriptional level, but also that this overexpression results in amplification of NPC1 protein levels and more successful NPC1 processing, ultimately leading to increased cholesterol clearance from the lysosomal compartments.
- The effects of arimoclomol in mutant NPC cells found across the in vitro studies are consistent, and downstream effects expected to result from the activation of a specific process in one study could be confirmed in another study to provide a cohesive picture of the mechanism of action of arimoclomol.

Figure 4: Arimoclomol Enhances the Binding of TFE3 to CLEAR Elements in Target Gene Promoters in Healthy Human Fibroblasts



Expression levels of selected CLEAR network genes after treatment with arimoclomol assessed by ChIP-qPCR assay.
For each gene promoter, ChIP data are presented as TFE3 immunoprecipitated DNA as % of input in control (PBS) or arimoclomol (400 µM, 84-86 hours) treated healthy human fibroblasts. *p<0.05, **p<0.01, ****p<0.0001.

Figure 5: Arimoclomol Increases Concentration of NPC1 Protein Across All Tested NPC1 Genotypes in Human NPC Fibroblasts



Data are presented as the mean change in % + SEM of NPC1 protein expression after arimoclomol treatment for 5 days, relative to PBS-treated control cells from a total of 3 to 5 independent experiments. NPC1 levels have been normalized to tubulin for 1061T/I1061T and P1007A/null, and to ponceau staining of total protein for the other cell lines. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6: Heatmap of Filipin Staining Intensity in Lysosomes Following Arimoclomol Treatment

Day	Arimoclomol Concentration (µM)					
	0	12	25	50	100	200
7	0%	-11%	-14%	-21%	-21%	-34%
14	0%	-17%	-27%	-25%	-32%	-55%
21	0%	-12%	3%	-15%	-29%	-55%
28	0%	-8%	-1%	-11%	-39%	-38%

Levels of unesterified cholesterol were measured by high-content imaging of filipin staining in NPC patient fibroblasts. Data represent the means of medians of 3 replicate experiments and are expressed as percent reduction in the staining intensities measured with different arimoclomol concentrations relative to vehicle control (0 µM) at Day 7, 14, 21 and 28 (the total cell line included >1400 cells for each condition). Colors range from green (lowest possible reduction of 100%) to white (cholesterol content observed with control) to red (symmetrically set to an increase of 100%) across arimoclomol concentrations by treatment day.

Methods and Statistics

Fibroblast cell lines derived from skin biopsies of clinically confirmed NPC patients and from healthy donors (wild-type controls) were maintained in culture in standard conditions (37°C and 5% CO_2, High glucose Dulbecco's Modified Eagle Medium supplemented with non-essential amino acids, 1% Penicillin-Streptomycin and 12% Fetal Bovine Serum). HeLa cells clone CCL-2 were acquired from American Type Culture Collection (ATCC) and cultured in the same medium and conditions as the fibroblast cell lines. As applicable, test solutions of arimoclomol citrate or U-18666A in PBS, or just PBS (vehicle control) were added to the cell medium. The effect of arimoclomol on the nuclear vs cytosolic distribution of TFEB and TFE3 was assessed in different cell types by immunofluorescence staining. Binding of TFEB/TFE3 to the promoter regions of CLEAR genes was assessed with chromatin immunoprecipitation (ChIP) and qPCR in wild-type fibroblasts treated with arimoclomol. The effects of arimoclomol on the expression levels of CLEAR genes were examined by qPCR in wild-type and NPC1 fibroblasts. Endo H assays and Western blotting were conducted to assess NPC1 protein level and maturation. High-content imaging of filipin staining was used to evaluate cholesterol clearance from lysosomes. All experiments were performed in the Orphazyme laboratory in Copenhagen, Denmark. Effects of treatment relative to control were assessed by a paired 2-tailed t-test or by repeated measures two-way analysis of variance (ANCOVA). Multiplicity was adjusted using Šídák's or Dunnett's multiple comparisons test. Expression levels of target genes relative to reference genes were calculated with the ΔΔCT-method. Analyses were performed using GraphPad Prism (versions 9.1.0, 9.1.1 or 9.3.1). Statistical significance was considered at a threshold value of 0.05.

Acknowledgements:
Orphazyme A/S sponsored and assumed overall responsibility for the in vitro studies presented. KemPharm acquired all assets and operations of Orphazyme, including arimoclomol, on June 01, 2022. Effective February 22, 2023, KemPharm changed its company name to Zevra Therapeutics (hereafter Zevra).

Disclosures:
HS is an employee and a stock owner of Zevra. TM and SG are stock owners and co-inventors of patents related to arimoclomol. CKF has received consulting fees from Zevra. TKJ is a co-inventor of patents related to arimoclomol and has received consulting fees from Zevra.